REMARKS

Claims 7-22 are active in the present application. Support for these claims is found in Claims 1-6 and the specification as originally filed, in particular, page 3, line 18 to page 4, line 19. No new matter is believed to have been added by these amendments.

Applicants wish to thank Examiner Lilling for the courteous telephonic discussion granted to the undersigned Applicants' representative on May 20, 2002. During this discussion, the Examiner requested clarification as to the differences between the claimed *E. coli* strains and the *E. coli* in McQuillen et al. In view of the foregoing amendments, and the following remarks, favorable reconsideration and allowance of all pending claims is requested.

With respect to the non-elected claims drawn to methods of producing arginine (Group III, see Claims 15-22), Applicants request that upon finding that the elected group is found to be allowable (Claims 7-14), the corresponding non-elected process claims should be rejoined in accordance with MPEP § 821.04.

The rejection of Claims 1 and 2 under 35 U.S.C. § 101 is obviated by the cancellation of these claims.

The rejection of Claims 1 and 2 under 35 U.S.C. § 112, first paragraph, is obviated by the cancellation of the claims.

As this rejection may apply to the present claims, Applicants note that deposit receipts for the deposited strains FERM BP-7925 and FERM BP-7926 were filed on June 22, 2001. These strains are specifically identified on page 8, lines 14-19 and page 9, lines 11-16. As noted on those pages, those strands have been deposited under the terms of the Budapest Treaty. In accordance with such deposit, Applicants submit that all restrictions imposed by

the depositor on the availability to the public of the deposited material will be irrevocably removed upon the granting of a patent.

Withdrawal of this rejection is requested.

The rejection of Claims 1 and 2 under 35 U.S.C. § 102(b) over McQuillen et al is obviated by the cancellation of the claims.

For the Examiner's reference, Applicants attach hereto a full copy of the McQuillen et al prior art reference[1].

The claimed *E. coli* strains claimed in Claims 7-14 are not the same as the McQuillen et al *E. coli* for the following reasons.

McQuillen et al discloses E. coli strain B, which is a wild type strain (see page 81, "Methods", line 1). There is nothing in McQuillen et al that discloses or suggests anything to the contrary and there certainly is nothing in McQuillen et al that discloses making a mutant *E. coli* strain or modify a wildtype *E. coli* so that it may utilize acetate as in Claim 7. Furthermore, the growth medium used to culture the *E. coli* strain contained significant amounts of glucose as the carbon source (see page 82, line 1), which is different from those bacteria claimed in, for example, Claims 8 and 9, which recite that the *E. coli* can grow on an agar medium using acetic acid or acetate as a sole carbon source.

Applicants point out that a wild type *E. coli* are unable to utilize acetic acid or acetate as the sole carbon source or which has been modified to utilize acetate. Applicants direct the Examiner's attention to *E. coli* strain 237 noted in the specification on page 8, line 14-19, which was unable to utilize acetic acid or acetate as a sole carbon source on an agar medium to grow or form colonies within two days at 37°C (see page 9, lines 1-6). In contrast, a mutant strain which was modified to have an ability to utilize acetate (e.g., strain 382), does

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[1] *J. Biol. Chem* vol. 207: pp 81-95 (1954).

have this ability. Furthermore, the wild type 237 strain was unable to utilize acetate as the sole carbon source in producing L-arginine compared to bacterial strains, which had been modified to utilize acetate as the sole carbon source, as evidenced in the specification in Table 2 on page 11, and Table 3 on page 12.

Therefore, it is clear that the *E. coli* of McQuillen et al is not the same as those *E. coli* claimed, and therefore the present claims are not anticipated by the disclosure of McQuillen et al. Withdrawal of this ground of rejection is requested.

Applicants submit that the present application is now ready for allowance. Early notification of such allowance is kindly requested.

Respectfully submitted,

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IN THE CLAIMS

--1-6. (Canceled).

7-22. (New).--

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CONTENTS OF VOLUME 207

No. 1, March, 1954

THE UTILIZATION OF ACETATE FOR SYNTHESIS IN ESCHERICHIA COLI

By KENNETH McQUILLEN* and RICHARD B. ROBERTS

(From the Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D. C.)

(Received for publication, May 29, 1953)

For many years the importance of the Krebs tricarboxylic acid cycle in bacterial metabolism has been contested. Some authors find evidence for the occurrence of the cycle in certain species of bacteria; in other cases, results are interpreted as proving that the cycle does not operate (see Ajl for a recent review (1)). In most of these studies, as indeed with those on other organisms, the emphasis has been on oxidation mechanisms, and few investigations have been carried out on growing cells. Abelson, Bolton, and Aldous (2, 3) studied the incorporation of C^{14}-labeled carbon dioxide into various components of *Escherichia coli* during active growth. Their data can be interpreted in terms of the tricarboxylic acid cycle functioning mainly in the synthesis of two groups of amino acids, one derived from glutamic acid and the other from aspartic acid.

It was of interest, therefore, to trace the flow of labeled atoms in other compounds which might be expected to give rise to derivatives which enter the cycle. C^{14}-Labeled glucose, amino acids, and acetic acid have now been used in this laboratory to study amino acid synthesis in *E. coli* during growth. The results so far obtained are, in general, in good agreement with the postulation of a tricarboxylic acid cycle and, moreover, permit a quantitative interpretation of the distribution of tracer atoms in a number of amino acids. A summary of this work and its interpretation is in press (4, 5).

The present paper deals with the incorporation of C^{14} from methyl- or carboxyl-labeled acetate into cellular material of *E. coli* growing exponentially under a variety of experimental conditions. Studies with other tracer molecules will be reported elsewhere.

Methods

E. coli strain B, as in earlier work reported from this laboratory, was the organism used (2, 3, 6).

The growth medium was the basal salts Medium C of the following composition: NH_4Cl 2 gm., Na_2HPO_4 6 gm., KH_2PO_4 3 gm., NaCl 3 gm., Mg 10 mg. (as $MgCl_2$), S 25 mg. (as Na_2SO_4), and H_2O to 1000 ml.

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Glucose was used at concentrations of 0.5 to 4.0 mg. per ml., and $C^{14}H_3COONa$, $CH_3C^{14}OONa$,[1] and other substances were added as described.

Growth Conditions—Cultures were grown overnight in Medium C containing 1 mg. of glucose per ml. with aeration at 37°. After harvesting and washing with Medium C, the cells were inoculated into a fresh glucose-salts medium and grown for about 2 hours to reach a steady exponential rate of growth. Such cells, after washing, would again grow exponentially immediately on incubation in the glucose-salts medium with or without further additions. Organisms pretreated in this way were used to insure reproducible behavior. An experimental growth period of 1 to 2 hours was used involving an increase of from 2- to 4-fold in the suspension density.

Growth was followed by making measurements at 650 mμ on a Beckman spectrophotometer, model DU, for which calibration curves had been prepared. Suspension densities are expressed in micrograms dry weight per ml.

Harvesting and Fractionation—After growth under the appropriate conditions, the organisms were centrifuged, washed with 0.85 per cent NaCl, and extracted successively as follows. 5 per cent trichloroacetic acid (TCA) for 30 minutes at 5° extracts Fraction 1 (the components of Fractions 1 to 5 are described below). 75 per cent ethanol for 30 minutes at 40–50° extracts Fraction 2, which is subdivided by addition of equal volumes of ether and water. The phases separate, leaving mainly alcohol-soluble protein in the aqueous phase (Fraction 2a) and lipide in the ether phase (Fraction 2b). A second ether addition yields more lipide, which is combined with Fraction 2b. Ether-75 per cent ethanol (1:1 volume per volume) for 15 minutes at 37° removes further fatty material and protein (Fractions 3a and b). 5 per cent TCA for 15 minutes at 100° extracts nucleic acids (Fraction 4). Ether washing of the residue leaves Fraction 5, which represents the main protein fraction of the organisms. For radioactivity measurements, aliquots of this material were dissolved in 0.1 N NaOH.

Radioactivity Measurements—Determinations were made on the bacterial culture at the beginning and end of each experiment, in the presence of NaOH or HCl, to assess total radioactivity and residual non-volatile radioactivity (*i.e.*, after removal of acetic acid).

Measurements were also made on the culture supernatant solution with NaOH or HCl, and on suspensions of the washed organisms. Each of Fractions 1 to 5 was assayed.

Counts were made on aliquots which were less (usually much less) than 1 mg. per sq. cm., so that self-absorption was less than 5 per cent.

[1] Supplied by Tracerlab; each 1 mc. per mM.

Chromatography—One- and two-dimensional ascending chromatograms were run on Whatman No. 1 paper. Three solvent systems were used: 70 per cent *sec*-butanol, 10 per cent formic acid, 20 per cent H_2O; 80 per cent phenol, 20 per cent H_2O, 0.3 per cent NH_3; and 70 per cent *tert*-butanol in 0.8 N HCl. Radioautographs of chromatograms were prepared on Kodak x-ray film.

Investigation of Fractions 1 to 5—Fraction 1. The cold TCA-soluble material, after two ether washes, was run in phenol on a one-dimensional chromatogram.

Fraction 2a. The alcohol-soluble protein was run on a one-dimensional chromatogram in both phenol and *sec*-butanol, and, after hydrolysis, on a two-dimensional chromatogram in this pair of solvents.

Fraction 2b. The lipide fraction was run on one-dimensional chromatograms in phenol and also *sec*-butanol.

Fraction 4. The nucleic acid fraction was hydrolyzed in 0.1 N HCl at 100° for 1 hour and, after removal of the acid, was run on a one-dimensional chromatogram in *tert*-butanol.

Fraction 5. The protein fractions were hydrolyzed in 6 N HCl in sealed tubes for 16 hours at 105°. The hydrolysates, after removal of acid, were run on two-dimensional chromatograms with *sec*-butanol, and then phenol as solvents.

The positions of radioactive amino acid spots were found by means of radioautographs and the radioactivities of these spots counted directly on the paper. In some cases spots were cut out from the paper chromatograms and eluted with water. The specific radioactivities of such samples were determined by assaying aliquots for amino acid content (with essentially the technique of Moore and Stein (7)) and also for radioactivity.

EXPERIMENTS AND RESULTS

Distribution of C^{14} in Major Fractions

As a result of a large number of experiments, it has been found that, in general, after 1 hour's exponential growth (*i.e.*, an approximate doubling of the suspension density) in the presence of $CH_3C^{14}OONa$ or $C^{14}H_3COONa$, the radioactivity is distributed as shown in Table I. In addition a small part of the acetate is converted to non-volatile compounds which are released into the medium.

Fraction 1 contains a wide variety of substances, but the major part of the radioactivity derived from labeled acetate is found in peptides containing glutamic acid. (The nature of the cold TCA-soluble fraction of *E. coli* has been investigated in this laboratory by means of other labeled substances; *i.e.*, $C^{14}O_2$, randomly labeled glucose, S^{35}, etc.)

Fraction 2a contains one or more alcohol-soluble proteins which have

also been investigated with other labeled compounds besides acetate. The fraction moves with the front in phenol, stays at the origin in *sec*-butanol, and is very difficult to elute from paper (see the alcohol-soluble protein described by Wynn and Rogers (8)). After hydrolysis, the material gives two-dimensional chromatograms with ninhydrin-positive spots corresponding to the usual range of amino acids, and with radioactivity in those same amino acids as are found labeled in the main protein Fraction 5 (see below). When cells were grown in the presence of randomly labeled glucose, all the radioactivity was associated with amino acids. Other experiments showed that one-sixth of the total protein of this organism was in this fraction.

Fraction 2b is mainly lipide, and the radioactivity moves with the front on chromatograms in either phenol or *sec*-butanol. No attempt has been made to fractionate further this material.

TABLE I

Distribution of C^{14} from $C^{14}H_3COONa$ and $CH_3C^{14}OONa$ in Major Fractions of E. coli

Fraction No.		Per cent total fixed radioactivity
1	Cold TCA	5
2a + 3a	Alcohol-soluble protein	8
2b + 3b	Lipide	34
4	Nucleic acid	4
5	Protein	49

Fraction 3 is roughly half lipide and half protein.

Fraction 4, the nucleic acid fraction, contains only a small amount of the total fixed radioactivity, and this appears from chromatograms of hydrolysates to be exclusively in the pyrimidines.

Fraction 5 accounts for about half of the radioactivity; it is the main protein fraction and, as will be seen, contains some nine labeled amino acids.

Effect of Acetate Concentration on Incorporation

Parallel cultures were grown in glucose-salts media supplemented with acetate at levels ranging from 2.5 to 550 γ per ml. After an approximate doubling in cell numbers, the organisms were harvested and fractionated. Fig. 1 shows the results of one such set of observations. The incorporation of C^{14} increases as the acetate concentration increases, but the distribution among the various fractions is constant.

At low acetate concentrations the radioactive acetate is appreciably diluted by acetate derived from glucose, and in view of this required correction the data cannot be used directly.

Time-Course of Acetate Fixation

Fig. 2 shows the time-course of acetate fixation by *E. coli*, with $C^{14}H_3COONa$ as a tracer. The total fixation and fixation into protein were measured after various intervals of growth. The observed values are plotted as points, while the curves represent plots of the expected uptake per mg. of cells calculated[2] from the equation:

$$\text{Uptake per mg. cells} = K(1 - e^{-at})$$

where a is taken from growth equation, $N = N_0e^{at}$.

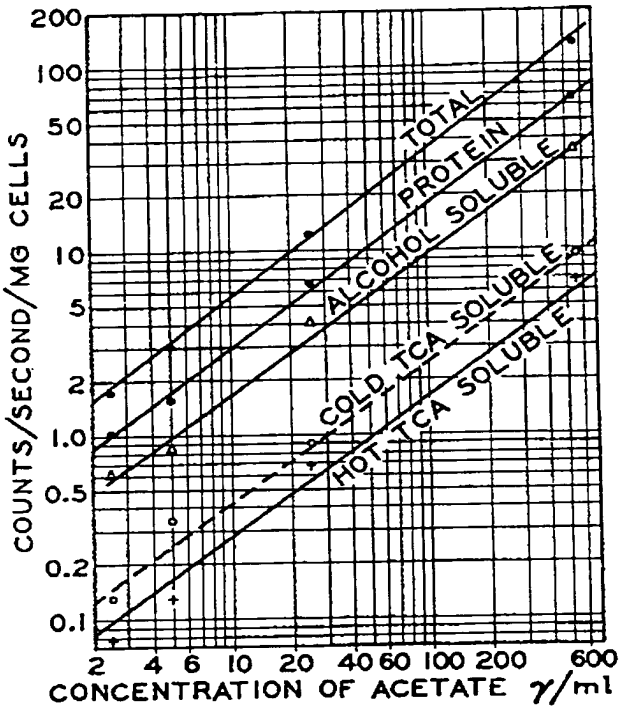

FIG. 1. Incorporation of $C^{14}H_3COOH$ by *E. coli* at various concentrations of added acetate.

The reasonably good agreement between the experimental points and the theoretical curves indicates that very little of the acetate is incorporated into fractions which have a rapid rate of turnover. This conclusion is supported by the finding that, in experiments in which the rate of loss of

[2] If the quantity of cells is increasing exponentially, the total quantity of cell at any time, N, is given by the expression $N = Ne^{at}$. The quantity of new cells formed in the interval from 0 to t is $N - N_0 = N_0(e^{at} - 1)$. If a tracer is added at $t = 0$, and the new cells incorporate a constant quantity of the tracer per mg. of cells, K, the total uptake will be $K(N - N_0) = KN_0(e^{at} - 1)$. The uptake per mg. of total cells (including both old and new) is then $KN_0(e^{at} - 1)/N_0e^{at} = K(1 - e^{-at})$. This equation applies only when there is no "turnover" and when there are no "metabolic pools."

radioactivity from labeled cells was measured, over 92 per cent of the activity remained in the organisms after 2 hours growth in a non-radioactive medium. As most of the acetate carbon is incorporated into protein and lipide, these results suggest that such substances are relatively stable in growing bacteria. On the other hand, when cells were deprived of an energy source, there was loss of radioactivity from the lipide fraction.

Fig. 2 also shows that, in the absence of a nitrogen source, the fixation of acetate is low, particularly into the protein. What little incorporation

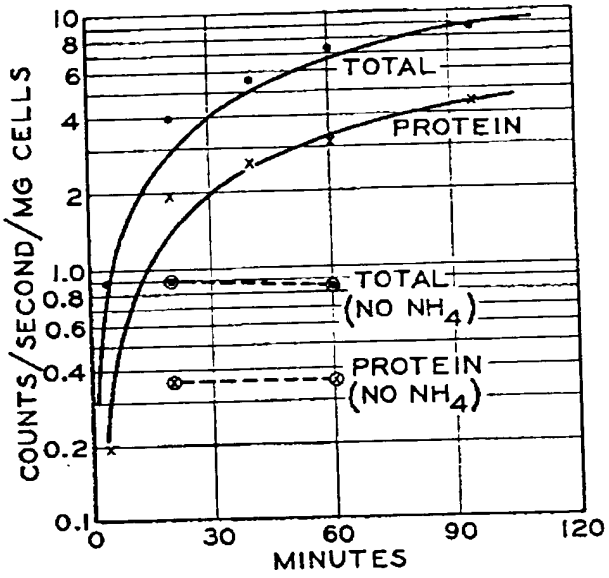

FIG. 2. Time-course of incorporation of $C^{14}H_3COOH$ by *E. coli*. Comparison of theoretical uptake (curve) with experimental values (points); solid lines with NH_4Cl.

does occur may be due to residual traces of ammonia since there is no increase with time.

Acetate Dilution

The dilution of added acetate by acetate formed from glucose can be demonstrated in a very direct fashion. Fig. 3 shows the time-course of incorporation of C^{14} from $CH_3C^{14}OOH$. Curve A shows that, when ample acetate is added (0.43 mg. per ml.), the observed uptake follows the expected curve. When radioactive acetate is added at a low level (0.021 mg. per ml.), the uptake (Curve B) falls below the expected curve as the radioactive acetate is both depleted and diluted. The quality of acetate formed from glucose is estimated to be 0.16 mg. per ml. at the end of 2 hours, which causes some dilution in Curve A but much greater dilution in Curve B.

CO_2 Fixation in Presence of Acetate

By the methods of Abelson *et al.* (2), the effect of inactive acetate on $C^{14}O_2$ fixation by *E. coli* was studied. Table II shows that acetate has virtually no effect on CO_2 fixation during growth, although higher concentrations of acetate inhibit growth.

Acetate Fixation in Major Fraction in Presence of Competitors[3]

The effects of a large number of substances on acetate incorporation have been studied. The presence of glutamic acid, aspartic acid, or leu-

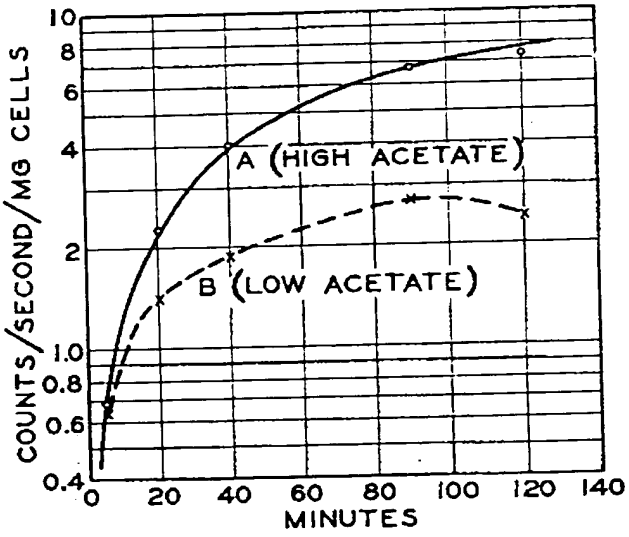

FIG. 3. Incorporation of $CH_3C^{14}OOH$ by *E. coli* at two concentrations of acetate. Curve A is drawn from the theoretical expression: uptake per mg. cells = $K(1 - e^{-at})$. The dotted curve is drawn to fit the experimental point. High acetate, 0.43 mg. per ml.; low acetate, 0.021 mg. per ml.

cine in the growth medium results in changes in the pattern of assimilation of C^{14} from $C^{14}H_3COONa$ into the major fractions of *E. coli*, as shown by the data in Table III.

Distribution of C^{14} among Amino Acids

Whenever *E. coli* has been grown in a glucose-salts medium with C^{14}-acetate (either methyl- or carboxyl-labeled) but with no further additions, it has been found that the following amino acids of the protein become labeled: glutamic acid, arginine, proline, aspartic acid, threonine, leucine, isoleucine, lysine, and methionine.

[3] The terms competitor, competition, etc., are used to indicate that certain compounds, which can be synthesized by *E. coli* when supplied externally, reduce the amount of endogenously formed material appearing in cell substance.

There is virtually no labeling of other amino acids, unless *E. coli* is adapted to grow on acetate as the sole carbon source, when, as is to be expected, all amino acids become labeled (9).

Table IV summarizes the amounts of radioactivity in various amino acids as counted directly on two-dimensional chromatograms of protein

TABLE II

Effect of Acetate on $C^{14}O_2$ Fixation during Growth

CH_3COONa concentration	Suspension density			C^{14} fixed per γ new growth
	Initial	Final	Increase	
γ per ml.	*γ per ml.*	*γ per ml.*	*γ per ml.*	*c.p.s.*
0	100	310	210	214
6	100	295	195	210
12	100	305	205	195
60	100	307	207	193
180	100	315	215	195
600	100	293	193	192
1800	100	295	195	190
6000	100	230	130	177

TABLE III

Competition with Acetate Fixation into Major Fractions

Experiment	Competitor	C^{14} fixed					
		Total	Cold TCA	Lipide	Alcohol-soluble protein	Hot TCA	Protein
B	None	100	100	100	100	100	100
C	Aspartic acid	80	70	92	53	49	85
D	Glutamic "	56	26	97	29	25	41
G	Leucine	84	86	93	58	115	100

E. coli grown for 90 minutes in the glucose-salts medium + 0.365 mM of $C^{14}H_3CO$-ONa. Amino acid supplements, 5.75 mM. The results are expressed as activities relative to 100 for the corresponding fraction of the control culture (Experiment B).

hydrolysates. The results are expressed as percentages of the activity of the glutamic acid.

There is good reason to believe that the amino acid composition of the protein of *E. coli* is essentially constant under the conditions used in these experiments. Abelson (unpublished) has devised a method for determining the relative proportions of most of the amino acids. The organisms are grown in randomly labeled glucose as the sole carbon source. The relative abundances are found by measuring the radioactivities of the amino acid spots on two-dimensional chromatograms of the protein hydrolysates from such cells. Table V is taken from Abelson's data.

By using such values it is possible to calculate relative specific activities for the amino acids shown in Table IV (see Table VI). Some of these values have been checked by elution of the amino acids from chromatograms and determination by the ninhydrin method of Moore and Stein (7). Results for relative specific activities obtained in this way are in good agreement with those shown in Table VI.

The specific activities of glutamic acid, proline, and arginine are so nearly equal as to suggest that these amino acids derive the same number

TABLE IV

Distribution of C^{14} in Various Amino Acids of E. coli

Experiment No.	Acetate concentration		Relative radioactivities							
	$C^{14}H_3COONa$, gm. per ml.	$CH_3C^{14}OONa$, gm. per ml.	Glu	Asp	Pro	Arg	Lys	Thr	Leu + Iso*	Leu†
B	30		100	27	39			13.5	60	46.5
1		30	100	15	38	43	14	6.5		
7		30	100	21	37			8.2		
KC4	500		100		40	40	20	14		
KC5		500	100	27	36	43	17	13		
A′		40	100	17	40	52		9.3	57	47.7
B′		40	100	19	45	50		9.0	72	63
C′		40	100	24	44	52		13	67	54
D′		40	100	32	44	52		17	67	50

Experiment 7, lysine (5.75 mM) present in the medium; Experiment A′, cells in lag phase; Experiment B′, early exponential; Experiments C′ and D′, cells in exponential phase. Growth at 37° except for Experiment D′, which was at 25°. The results are expressed as activities relative to 100 for glutamic acid in each experiment.

* Observed values of leucine + isoleucine.

† Estimated values of leucine obtained by subtracting observed value of threonine from observed value of leucine + isoleucine. Independent tests show that radioactivities of isoleucine and threonine are approximately equal (5).

of carbon atoms from acetate. Similarly there appears to be a relationship between aspartic acid and threonine. The results of experiments in which *E. coli* was grown in the presence of labeled acetate and a single inactive amino acid further emphasize these groupings (Table VII). Most of the results shown were obtained with both methyl- and carboxyl-labeled acetate.

Aspartic acid reduces incorporation of acetate carbon into the aspartic acid, threonine, lysine, and methionine of the protein by 75 to 80 per cent. Glutamic acid on the other hand abolishes virtually all acetate uptake into amino acids with the exception of leucine, the activity of which is unchanged.

Each of the amino acids, proline, arginine, lysine, leucine, and methio-

nine, is capable of almost complete suppression of acetate incorporation into itself but has no effect on other amino acids. The relative specific

TABLE V

*Relative Abundance of Some Amino Acids in Protein of E. coli**

Amino acid	Relative molar abundance
Glutamic acid	100
Aspartic "	97.5
Proline	37.8
Arginine	41.5
Lysine	47.7
Threonine	42.4
Probable error	5%
Leucine	67
Isoleucine	40
Methionine	27
Probable error	10%

* Unpublished data of Abelson.

TABLE VI

Relative Specific Activities of Some Amino Acids of E. coli Grown in $C^{14}H_3COONa$ or $CH_3C^{14}OONa$

Experiment No.	Glu	Asp	Pro	Arg	Lys	Thr	Leu
B	100	28	102			32	70
1	100	16	101	103	27.5	15.2	
7	100	21	98			19	
KC4	100		105	97	42	33.5	
KC5	100	27.5	95	105	36	30.8	
A′	100	17.4	106	125		21.9	72
B′	100	19.4	119	120		21.3	95
C′	100	25	116	125		31.4	81
D′	100	33	116	125		40.3	75

The results are expressed as specific activities relative to 100 for glutamic acid in each experiment; derived from Tables IV and V.

radioactivities of some of the amino acids have been determined in the absence and presence of competitors, as shown in Table VIII.

Under a variety of conditions it has been found that proline has almost exactly the same specific activity as that of glutamic acid. This is true even in Experiment D of Table VIII, where, in the presence of added glu-

tamic acid, incorporation of acetate into glutamic acid (and all other amino acids except leucine) is reduced to less than 5 per cent of normal. Of course, when proline itself is present as competitor, acetate incorporation proceeds normally into glutamic acid but is specifically suppressed as far

TABLE VII

Incorporation of C^{14}-Acetate into E. coli Proteins in Presence of C^{12}-Amino Acids in Medium

C^{12}-Amino acid added to medium	Amino acids in protein hydrolysate which have incorporated C^{14}						
	Proline	Arginine	Leucine	Lysine	Aspartic	Glutamic	Threonine
None (control)	+	+	+	+	+	+	+
Proline	−	+	+	+	+	+	+
Arginine	+	−	+	+	+	+	+
Leucine	+	+	−	+	+	+	+
Isoleucine	+	+	+	+	+	+	+
Lysine	+	+	+	−	+	+	+
Aspartic acid	+	+	+	−	−	+	−
Glutamic "	−	−	+	−	−	−	−

The synthetic medium contained C^{12}-glucose and $C^{12}O_2$. Either $C^{14}H_3COOH$ or $CH_3C^{14}OOH$ was added, both leading to identical results. A minus sign indicates that the incorporation of C^{14} is strongly suppressed as compared to the control case.

TABLE VIII

Relative Specific Activities of Some Amino Acids in Presence of Amino Acid Competitors

Experiment	Competitor	Glutamic acid	Aspartic acid	Proline	Threonine	Leucine
B	None	100	28	102	32	70
C	Aspartic acid	100	6	107	8	101
G	Leucine	100	28	103	31	2.65
D	Glutamic acid	100	53	106	87	2500

The results are expressed as specific activities relative to 100 for glutamic acid in each experiment. The absolute specific activities of leucine in Experiments B, C, and D are approximately equal.

as proline is concerned. Aspartic acid and threonine bear the same relationship to each other as to glutamic acid and proline.

Acetate Fixation during Different Phases of Growth

An experiment was carried out to determine the influence of the state of the cells and the temperature of growth on the distribution of acetate carbon in the amino acids of *E. coli*. Cells from an overnight culture which

had reached the stationary phase were washed, and one sample was incubated immediately in a glucose-salts medium containing tracer acetate. Other samples were grown for 1 and 2 hours before being washed and incubated in the radioactive medium. The three cultures were in lag, early exponential, and exponential phases respectively during incubation for 1 hour with the tracer. A second sample of cells in the exponential phase was grown for 2 hours at 25° instead of the usual 37°. Table IV shows the distribution of radioactivity in various amino acids (Experiments A′, B′, C′, and D′).

Although the ratio of radioactivity in glutamic acid to that in aspartic acid varies under different conditions of growth, the ratios, glutamic acid-

TABLE IX

Oxidation of Acetate

	(*A*)	(*B*)
1. SN (non-volatile), c.p.s. per 100 ml.	5.6	11.5
2. Cells, c.p.s. per 100 ml.	38	4.2
3. CO_2, c.p.s. per 100 ml.	7.45	6.17
4. Total CO_2 produced, mM per 100 ml.	0.73	0.34
5. Glucose consumed, mM per 100 ml.	0.26	0.10
6. Total acetate ((1) + (2) + (3)), c.p.s. per 100 ml.	51.0	21.8
% as CO_2 (3:6)	14.6	28.3
7. Average density of cells, γ per ml.	145	167

Initial concentrations, glucose, 0.77 mg. per ml.; acetate, 0.5 mg. per ml.; NH_4Cl (*A*), 1.5 mg. per ml., and (*B*), 0 mg. per ml.

proline, glutamic acid-arginine, and aspartic acid-threonine are relatively constant.

Oxidation of Acetate

The quantity of acetate converted to CO_2 is small and cannot be measured accurately by the usual technique of incubating the cells with aeration. To measure the CO_2 production, the cells were shaken in sealed bottles for 1 hour. At the end of the hour HCl was added to release the CO_2 from the fluid, the fluid was removed, and the CO_2 was trapped by adding 0.1 N NaOH. $BaCO_3$ was then precipitated and measured. Table IX shows that, when the cells are growing in the presence of glucose and NH_4^+, the ratio of C^{14} incorporated to C^{14} released as $C^{14}O_2$ is 7:1. This is in agreement with predictions based on the Krebs cycle (4). When NH_4^+ is omitted, there is very much less incorporation of acetate, more is converted to non-volatile compounds in the medium, and a larger fraction is converted to CO_2. The quantity of acetate converted to all other prod-

ucts is reduced by a factor of 2, which agrees with other observations that, in the absence of NH_4^+, the utilization of glucose, uptake of O_2, and production of CO_2 all drop by a factor of 2. Evidently the rate of flow in the tricarboxylic acid cycle is reduced in the same ratio as other processes for degrading glucose. Other experiments show that, when neither glucose nor NH_4^+ is present, 90 per cent of the acetate acted on by the cell is converted to CO_2.

DISCUSSION

The nature of two of the fractions isolated from *E. coli* by the extraction procedure adopted here is worth comment. Cold 5 per cent TCA liberates a number of small molecular weight substances, including peptides. When cells are grown in the presence of labeled acetate, glutamic acid peptides account for most of the radioactivity in this fraction. The 75 per cent ethanol extract is of interest in that it contains protein as well as lipide. It is not known whether these exist as lipoprotein in the intact cell, but, if so, they are readily resolved after extraction.

In growing organisms there is parallel incorporation of acetate carbon into all cell fractions over a wide range of acetate concentration (Fig. 1) and the total fixation and fixation into protein follow the time-course predicted from the growth equation (Fig. 2). Also there is little turnover of C^{14}, once it has been assimilated. Since most of the fixed acetate carbon occurs in protein and lipide, it appears that these substances are relatively permanent once formed, or else are degraded and synthesized without loss of radioactivity. Similar results have been obtained with $C^{14}O_2$ and $S^{35}O_4^=$. In our experiments there is little assimilation of acetate in the absence of a nitrogen source (contrast the findings reported by Clifton (10)).

The amount of acetate fixed by *E. coli* is reduced if either aspartic acid, or, more effectively, glutamic acid is present in the growth medium (Table III). Neither of these substances has much effect on the incorporation into lipide; both reduce fixation into protein, and glutamic acid has a marked effect on the cold TCA-soluble fraction, the main labeling of which occurs in the glutamic acid peptides. The radioactivity fixed into the nucleic acids is also reduced by both glutamic and aspartic acids, indicating a pathway from aspartic acid to the pyrimidines.

Perhaps the most interesting aspect of the present study is the light it throws on amino acid synthesis in *E. coli*. When this organism is grown in a glucose-salts medium containing labeled acetate, the amino acids of the protein fall into two classes, those which become labeled and those which do not (11). This by itself suggests that the carbon chains of certain amino acids are formed from intermediates lying between glucose and pyruvic acid of the Embden-Meyerhof sequence or of some other degradative

pathway of glucose, whereas the other amino acids involve incorporation of acetate carbon at some stage in their synthesis. This latter group can be divided into three: (*a*) glutamic acid, proline, and arginine; (*b*) aspartic acid, threonine, lysine, methionine, and isoleucine; and (*c*) leucine.

This classification is based among other things on the results of competition experiments and comparisons of specific activities. For example, glutamic acid, proline, and arginine have specific activities very nearly identical to each other, as do aspartic acid and threonine. The presence of aspartic acid in the growth medium eliminates the uptake of C^{14} from radioactive acetate into its family of amino acids, glutamic acid into its family, and also into the aspartic acid family. Leucine alone of those amino acids labeled from acetate is not affected by aspartic or glutamic acid. Each of the amino acids mentioned prevents the incorporation of tracer into itself.

It is suggested that two separate acetate fixation mechanisms are involved in amino acid synthesis, one in which leucine is a product and a second in which glutamic acid (or a substance in equilibrium with it, such as α-ketoglutaric acid) is an early product. Some of the arguments in favor of glutamic acid rather than aspartic acid, or both, occupying this position are as follows: Aspartic acid does not compete with acetate incorporation into the glutamic acid family. Glutamic acid does suppress acetate fixation into the aspartic acid group. Moreover, specific activities of the glutamic acid group are 3 to 6 times as high as those of the aspartic acid family. These findings render unlikely a dicarboxylic acid cycle involving acetate condensation to succinate, since this would result in high specific activity of aspartic acid and would tend to suppress the incorporation of $C^{14}O_2$.

The obvious resemblance of Table VII to the tables in papers on "biochemical mutants" is striking. Biosynthetic pathways from glutamic acid to proline (12), glutamic acid to arginine (12), aspartic acid to threonine (3) via homoserine, which may be the source of the C-4 chain of methionine (13), and a relationship between threonine and lysine (14) have been postulated for various microorganisms. It seems likely that in *E. coli* there exist all of these interrelations.

Further evidence, based on the use of labeled carbon dioxide, glucose, and amino acids (4, 5), supports the present findings and suggests that glutamic acid (or a substance in equilibrium with it) supplies all 5 carbon atoms of proline and all but the amidine carbon of arginine, and that aspartic acid (or a substance in equilibrium with it) supplies 4 carbon atoms of threonine, lysine, methionine, and isoleucine.

Roberts *et al.* (4) give a theoretical treatment of the quantitative flow of carbon atoms into the glutamic acid and aspartic acid families based on

the Krebs tricarboxylic acid cycle. This treatment, once the flows are determined, predicts the positions and specific radioactivities in the amino acids of particular tracer carbon atoms derived from various metabolites. It is useful in that, if the flows are determined experimentally by using, for example, $C^{14}O_2$, then predictions for labeled acetate or glucose-1-C^{14} can be made and verified.

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SUMMARY

1. The incorporation of radioactive acetate into various fractions of actively growing *Escherichia coli* has been studied in the absence and presence of certain amino acids.

2. In a glucose-salts growth medium, *E. coli* fixes both acetate carbons into glutamic acid, proline, arginine, aspartic acid, threonine, lysine, methionine, isoleucine, and leucine.

3. Specific activity measurements and competition experiments indicate biosynthetic relationships among most of these amino acids.

4. These and other results can be interpreted in terms of the Krebs tricarboxylic acid cycle, functioning in actively growing *E. coli* mainly as part of a mechanism for synthesis of a group of amino acids.

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